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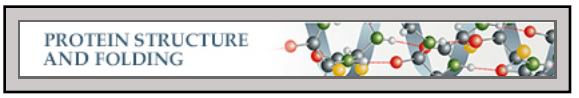

Identification of *S*-Hydroxylysyl-methionine as the Covalent Cross-link of the Noncollagenous (NC1) Hexamer of the α1α1α2 Collagen IV Network: A ROLE FOR THE POST-TRANSLATIONAL MODIFICATION OF LYSINE 211 TO HYDROXYLYSINE 211 IN HEXAMER ASSEMBLY

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A ROLE FOR THE POST-TRANSLATIONAL MODIFICATION OF LYSINE 211 TO HYDROXYLYSINE 211 IN HEXAMER ASSEMBLY*

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Collagen IV networks are present in all metazoans as components of basement membranes that underlie epithelia. They are assembled by the oligomerization of triple-helical protomers, composed of three α-chains. The trimeric noncollagenous domains (NC1) of each protomer interact forming a hexamer structure. Upon exposure to acidic pH or denaturants, the hexamer dissociates into monomer and dimer subunits, the latter reflect distinct interactions that reinforce/cross-link the quaternary structure of hexamer. Recently, the cross-link site of the α1α1α2 network was identified, on the basis of x-ray crystal structures at 1.9-Å resolution, in which the side chains of Met^{93} and Lys^{211} were proposed to be connected by a novel thioether bond (Than, M. E., Henrich, S., Huber, R., Ries, A., Mann, K., Kuhn, K., Timpl, R., Bourenkov, G. P., Bartunik, H. D., and Bode, W. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 6607–6612); however, at the higher resolution of 1.5 Å, we found no evidence for this cross-link (Vanacore, R. M., Shanmugasundararaj, S., Friedman, D. B., Bondar, O., Hudson, B. G., and Sundaramoorthy, M. (2004) *J. Biol. Chem.* 279, 44723–44730). Given this discrepancy in crystallographic findings, we sought chemical evidence for the location and nature of the reinforcement/cross-link site. Trypsin digestion of monomer and dimer subunits excised a ~5,000-Da complex that distinguished dimers from monomers; the complex was characterized by mass spectrometry, Edman degradation, and amino acid composition analyses. The tryptic complex, composed of two peptides of 44 residues derived from two α1 NC1 monomers, contained Met^{93} and Lys^{211} post-translationally modified to hydroxylysine (Hyl^{211}). Truncation of the tryptic complex with post-proline endopeptidase reduced its size to 14 residues to facilitate characterization by tandem mass spectrometry, which revealed a covalent linkage between Met^{93} and Hyl^{211}. The novel cross-link, termed *S*-hydroxylysyl-methionine, reflects at least two post-translational events in its formation: the hydroxylation of Lys^{211} to Hyl^{211} within the NC1 domain during the biosynthesis of α-chains and the connection of Hyl^{211} to Met^{93} between the trimeric NC1 domains of two adjoining triple-helical protomers, reinforcing the stability of collagen IV networks.

Collagen IV networks are components of basement membranes that underlie epithelia, compartmentalize tissue, and influence cell behavior. The networks are assembled from a family of six polypeptide chains (α1–α6) that associate forming three subtypes of triple-helical protomers with distinct chain compositions: α1α1α2, α3α4α5, and α5α5α6 (1, 2). The protomers self-assemble by end-to-end associations in which the amino termini of four protomers associate tail-to-tail forming the 7 S domain, and the carboxyl termini of two protomers associate head-to-head through the noncollagenous (NC1)[1] domains, forming dimers. At the interface of the head-to-head connection, the trimeric NC1 domains exist as a hexamer, a stable complex that can be excised by cleavage with collagenase for *in vitro* studies.

The NC1 domain plays a pivotal role in the assembly of the distinct collagen IV networks. In protomer assembly, the NC1 domains (monomers) of three chains interact, forming a NC1 trimer, to select and register chains for triple-helix formation. In the network assembly, the NC1 trimers of two protomers interact, forming a NC1 hexamer structure, to select and connect protomers. Upon exposure to acidic pH or denaturants, isolated NC1 hexamer dissociates into monomers and dimers, the latter reflecting the presence of cross-links that stabilize the trimer-trimer interface. The cross-links connect α1-like monomers (α1–α1, α1–α5, and α3–α5) and α2-like monomers (α2–α2, α2–α6, and α4–α4) (3, 4). For two decades, the reduc-

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[1] The abbreviations used are: NC1, non-collagenous domain; PBM, placenta basement membrane; GdnHCl, guanidine hydrochloride; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; LC-ESI/MS/MS and LC-ESI/MS^3, liquid chromatography electrospray tandem mass spectrometry; HPLC, high performance liquid chromatography; DTT, dithiothreitol; Hyl, hydroxylysine; MS^3, third fragmentation mass spectrometry.

ible dimers were thought to consist of monomers bound by disulfide cross-links (5, 6). However, the recent x-ray crystal structures of the NC1 hexamers of bovine lens capsule basement membrane and human placenta basement membrane, determined independently by us (7) and Than *et al.* (8), respectively, have disproved this hypothesis. An alternative explanation was proposed by Than *et al.* (8) in which the cross-link is a thioether bond between Met^{93} and Lys^{211} that bridges the trimer-trimer interface; the evidence was based on electron density maps at 1.9-Å resolution, suggesting the existence of both cross-linked and noncross-linked residues at this site (8). However, in a subsequent study at the higher resolution of 1.5 Å, we found no evidence for this cross-link (9).

Given this discrepancy in crystallographic findings, we sought chemical evidence in the present study for the location and nature of the reinforcement/cross-link site. We used tryptic digestion in combination with mass spectrometry as a strategy to search for post-translational modifications that may go undetected by crystallography. The results revealed that the site is located at the trimer-trimer interface of the NC1 hexamer, characterized by a novel covalent cross-link: *S*-hydroxylysylmethionine. The cross-link is uniquely labile to conditions typically used for characterization, rendering it a challenge for detection. The findings are the first report of chemical evidence for the location and nature of the reinforcement/cross-link site, and the presence of Hyl within the NC1 domain.

EXPERIMENTAL PROCEDURES

Materials—Bovine placenta was purchased from Pel-Freeze Biologicals (Rodgers, AR). PBM NC1 hexamer was prepared by collagenase digestion as previously described (10). Bacterial collagenase (CLSPA) was purchased from Worthington (Lakewood, NJ).

Separation of Monomers and Dimers—Monomers and dimers of the NC1 domain were isolated as described elsewhere with minor modifications (10). Briefly, PBM hexamers (5 mg) were denatured in 0.2 M Tris-HCl, pH 8.5, buffer containing 4 M GdnHCl and 25 mM DTT and incubated in a boiling water bath for 20 min. Subsequently, the reduced proteins were alkylated with 50 mM iodoacetamide in the dark for 30 min at room temperature. To fractionate dimer and monomer subunits, the denatured hexamer sample was run through a Sephacryl S-300 column (120 × 2.5 cm) that had been equilibrated in 50 mM Tris-HCl, pH 7.4, buffer containing 4 M GdnHCl. The fractions with higher absorbance at 280 nm were analyzed by 4–20% linear gradient SDS-PAGE (Bio-Rad). The fractions containing monomer or dimers were pooled, concentrated, and washed with 50 mM ammonium bicarbonate, pH 7.8, buffer in Amicon Ultra filters 10,000 MWCO (Millipore Corp. Bedford, MA). In addition, to investigate the effect of DTT and denaturant as a function of temperature and time of incubation on hexamer dissociation, the chromatography analyses were performed on a TSK SW_{xl}3000 ToSo-Hass HPLC column equilibrated with 50 mM Tris-HCl, pH 7.5, containing 4 M GdnHCl, and connected to an ÄKTA Purifier HPLC chromatography system (Amersham Biosciences). Gels were stained with Bio-Safe colloidal Coomassie Brilliant Blue G-250 (Bio-Rad) for MS compatibility.

Separation of Tryptic Peptides by Size-exclusion Chromatography—NC1 dimers and monomers (reduced and alkylated) were separately incubated with sequencing grade-modified trypsin (Promega, Madison, WI) at a ~1:25 enzyme to protein ratio at room temperature for 16 h. Tryptic peptides were then separated in a Superdex™ peptide column (Amersham Biosciences) equilibrated with 50 mM ammonium bicarbonate, pH 7.8, and calibrated with small peptides. The elution of peptides was monitored by absorbance at 230 nm. The approximate molecular weight of the T_{5k}-complex was estimated by comparing its elution time to elution time of peptide standards.

Truncation of the T_{5k}-complex with Post-proline Endopeptidase—The T_{5k}-complex, isolated by gel filtration peptide column, was further truncated with post-proline endopeptidase from *Flavobacterium meningsepticum* (Seikagaku America, East Falmouth, MA) in 0.1 M ammonium bicarbonate, pH ~ 7.8, for 3 h at 37 °C. The products of the digestion were immediately analyzed by LC-ESI/MS (see below).

MALDI-TOF MS—Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was performed on a Voyager 4700 mass spectrometer or on a Perceptive Biosystems Voyager Elite (Applied Biosystems, Foster City, CA). Tryptic peptides derived from the T_{5k}-complex were prepared by the dried-droplet method using α-cyano-4-hydroxycinnamic acid as a matrix. α-Cyano-4-hydroxycinnamic acid was dissolved in water/acetonitrile/trifluoroacetic acid (50:49.9:0.1) at a concentration of 10 mg ml^{-1}. The peptides were initially identified by comparing the experimental masses of each peak with computer-predicted masses of tryptic peptides from the bovine NC1 domain (IV) sequences (7). Identity of some peptide sequences was confirmed by inducing ion fragmentation using the instrument in the tandem mode (MS/MS).

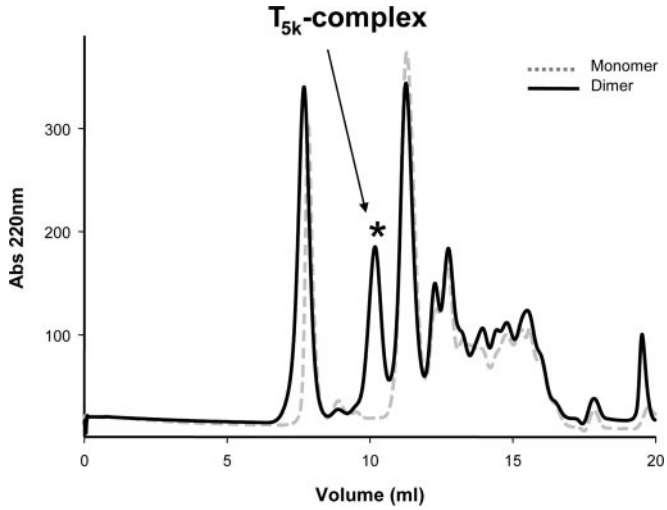

FIG. 1. **Trypsin digestion reveals a structural difference between dimer and monomer subunits α1 NC1 domains.** NC1 dimers and monomers were dissociated from bovine PBM NC1 hexamer in 4 M GdnHCl, reduced, and alkylated, and then fractionated by gel-filtration chromatography (Fig. 6, *panel b*). The dimer and monomer subunits were separately digested with trypsin, and the tryptic products were fractionated by size-exclusion chromatography on a Superdex peptide column as described under "Experimental Procedures." The fractionation was monitored by measurement of absorbance at 220 nm for monomers (*dashed line*) and dimers (*solid line*). The *asterisk* indicates the retention time of a tryptic (*T*) complex(s) of ~5000 molecular weight that distinguishes dimers from monomers.

LC-ESI/MS/MS and LC-ESI/MS^3—The LC-ESI/MS/MS and LC-ESI/MS^3 analyses were performed on a ThermoFinnigan LTQ linear ion trap mass spectrometer equipped with a ThermoFinnigan Surveyor LC pump, microelectrospray source, and Xcalibur 1.4 instrument control and data analysis software. HPLC separation of the NC1 tryptic peptides was achieved with a C_{18} capillary column at 0.7 ml min^{-1} flow rate. Solvent A was H_2O with 0.1% formic acid and solvent B was acetonitrile containing 0.1% formic acid. The gradient program was: 0–3 min, linear gradient 0–5% B; 3–5 min, 5% B, 5–50 min, linear gradient to 50% B; 50–52 min, linear gradient to 80% B; 52–55 min, linear gradient to 90% B; 55–56 min, 90% B in solvent A. A blank sample (0.1% formic acid) was run between the two analyses. Each sample was subjected to two LC-ESI/MS/MS analyses. In the first analysis, MS/MS spectra of the peptides were obtained using data-dependent scanning in which one full MS spectrum (mass range 400–2000 atomic mass units) was followed by three MS/MS spectra. In the second run, several specific precursor masses were selected for MS/MS analysis in a targeted fashion. For the LC-ESI/MS^3 analyses, the MS^3 spectra were obtained using data-dependent scanning in which one full MS spectrum is followed by one MS/MS spectrum. The three most intense ions in the MS/MS spectrum were selected for a third fragmentation (MS^3).

Software for Sequence and Post-translational Modification Analysis—Samples of trypsin-digested monomers and dimers were analyzed using LC-MS/MS. The data base search algorithm SEQUEST was used to identify peptides from the fragment ions recorded in the tandem mass spectrum. P-Mod (11), a statistics based algorithm, allowed for the successful identification of hydroxylation sites.

Analytical RP-HPLC of the T_{5k}-complex—The T_{5k}-complex peak was analyzed on an ÄKTA Purifier liquid chromatography system run by UNICORN 4.11 software (Amersham Biosciences). Runs were performed on a Supelcosil LC-318 reversed-phase analytical HPLC column

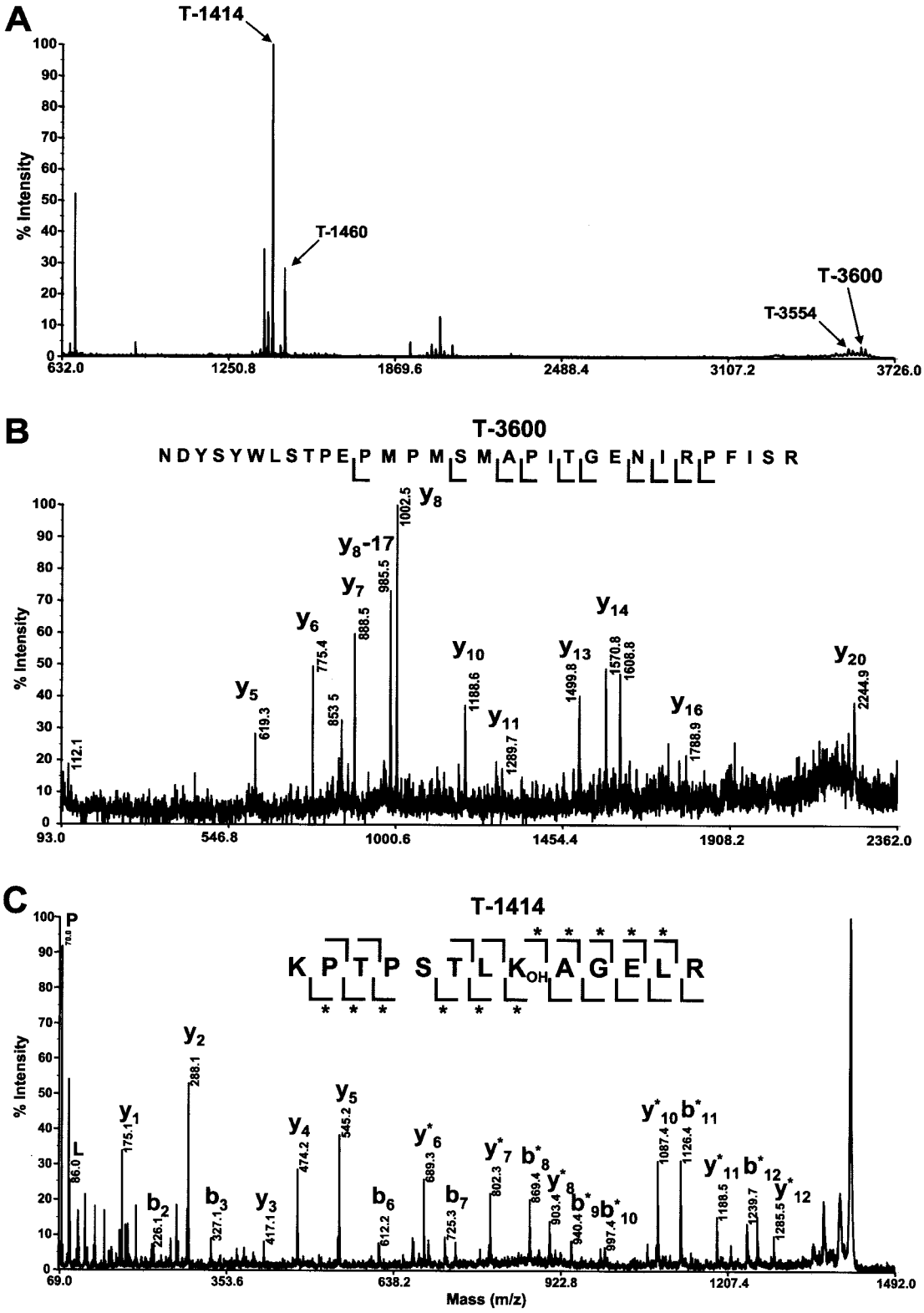

FIG. 2. **MALDI-TOF MS analyses of T_{5k}-complex reveals the presence of 2 component peptides (T-1414 and T-3600) and a post-translational modification of lysine to Hyl.** *Panel A*, MALDI-TOF mass spectrum of the T_{5k}-complex shown in Fig. 1. *Arrows* indicate ions at m/z 1414 and 3600 selected for MALDI-TOF/TOF tandem MS, the fragmentation spectra for which are shown in *panels B* and *C*, respectively, where *b* (⎡) and *y* (⎣) ions are denoted along with internal fragmentation ions.

(Sigma). The samples were loaded into the column that had been equilibrated with buffer A (95% water with 0.1% trifluoroacetic acid and 5% acetonitrile) with a flow rate of 1 ml/min. The peptides were eluted with a linear gradient up to 40% buffer B (0.085% trifluoroacetic acid, 95% acetonitrile and water) over 60 min. Peptide elution was monitored by absorbance at 215 nm.

Amino Acid Composition Analyses—Amino acid analysis was carried out on the T-5014 complex at the W.M. Keck Facility at Yale University (New Haven, CT) on a Beckman model 7300 ion-exchange instrument following a 16-h hydrolysis at 115 °C in 100 μl of 6 N HCl, containing 0.2% phenol. After hydrolysis, the HCl was dried in a SpeedVac, and the resulting amino acids dissolved in 100 μl of Beckman sample buffer. The instrument was calibrated with a 2-nmol mixture of amino acids and was operated via the manufacturer's programs and with the use of

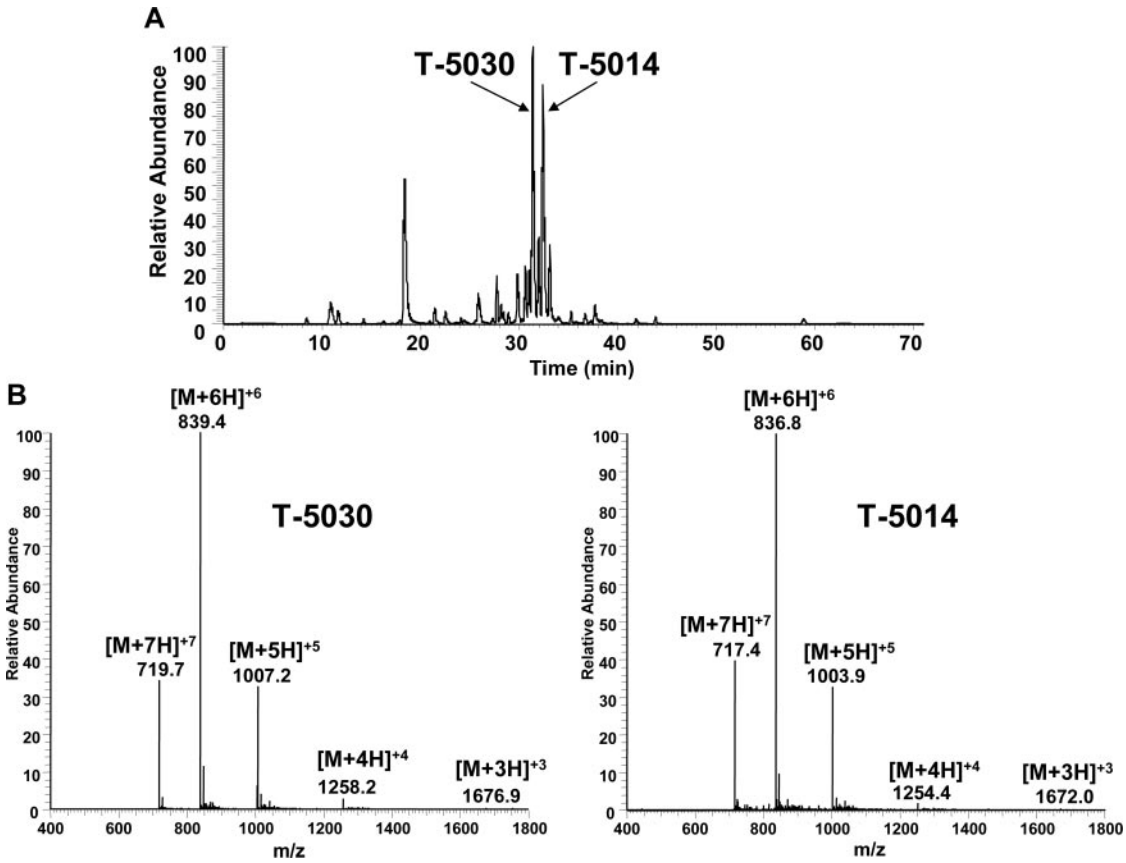

FIG. 3. **T-1414 and T-3600 peptides exist as a stable binary complex (T-5014 and T-5030) as determined by LC-ESI/MS^3 analysis.** *Panel A* shows the total ion current trace for the chromatographic separation on a C_{18} stationary phase of the T_{5K}-complex (Fig. 1). *Panel B*, full scan ESI/MS/MS spectra reveals that T_{5K}-complex display two main components: complexes T-5014 and T-5030. Multiply charged ions of the peptide complexes are indicated *above* the corresponding peaks.

their buffers. Data analysis was carried out on an external computer using PerkinElmer/Nelson data acquisition software.

Edman Degradation Sequencing—NH_2-terminal peptide sequencing of the T-5014 complex was carried out at the W.M. Keck Facility at Yale University on an Applied Biosystems Procise 494 cLC instrument equipped with on-line HPLC for the identification of the resulting phenylthiohydantoin-derivatives.

RESULTS

Identification of a Structural Difference between Dimers and Monomers of the NC1 Domain: Isolation of the Reinforcement Site by Trypsin Digestion—Trypsin digestion was used to identify structural differences between monomer and dimer subunits of the NC1 hexamer domain of collagen IV from bovine placenta basement membrane. To isolate monomers and dimers, the NC1 hexamers were reduced and alkylated in 4 M GdnHCl, then fractionated on a gel filtration column equilibrated with 4 M GdnHCl (Fig. 6, Ref. 9). The dimers and monomers were separately digested with trypsin under conditions for maximal cleavage. The peptide products were then fractionated over a size-exclusion chromatography column (Fig. 1). A comparison of the profiles for monomer (dotted) and dimer (solid) reveals that dimers yield an extra peak (M_r = ~5000), and therefore are designated as T_{5k}-complex, indicative of a structural distinction between dimer and monomer subunits.

The chemical nature of the T_{5k}-complex was characterized by mass spectrometry. Because of the large size of the T_{5k}-complex, the MALDI-TOF MS instrument was optimized to detect ions in the *m/z* range 500–8000. Surprisingly, the most intense ion in the spectrum was *m/z* 1413.8 and the highest mass observed was *m/z* 3601.6 (Fig. 2). The mass spectra were identical in linear or reflectron mode, which rules out a possible in-source fragmentation. Although the T_{5k}-complex was not observed, the combined mass of T-1414 plus T-3600 peptides is ~5014, a value close to that observed by size-exclusion chromatography.

To identify the sequence of T-1414 and T-3600 peptides, they were analyzed by MALDI-TOF-TOF tandem MS. Fig. 2*B* shows the fragmentation spectrum for the T-3600 peptide, which is consistent with the peptide sequence, ^{77}NDYSYWLSTPEPMPMSMAPITGENIRPFISR^{107}, derived from the α1 NC1 domain. The mass of the T-1414 peptide was not predicted from the α1 NC1 domain sequence, therefore, it was considered to be a modified peptide. As shown in Fig. 2*C*, the fragmentation pattern of the T-1414 peptide is consistent with the sequence, ^{204}KPTPSTLKAGELR^{216}, but with 16 extra mass units attached to Lys^{211}. It is well known that lysine residues within the collagenous domain of collagen IV are post-translationally modified by a lysyl hydroxylase catalyzed addition of a hydroxyl group (12), converting lysine to 5-hydroxylysine (Hyl). Thus, the extra 16 mass units observed in the T-1414 peptide in comparison to the known primary sequence, suggests the presence of Hyl^{211} (confirmed below). Therefore, the results are consistent with a stable complex (T-5014), composed of T-1414 and T-3600 peptides, which dissociate before or during sample preparation for MALDI-TOF MS analyses.

To test the hypothesis that T-1414 and T-3600 peptides form a stable complex, we analyzed the T_{5k}-complex, isolated by gel-filtration (Fig. 1), using LC-ESI/MS/MS. As shown in Fig. 3, the sample displayed two major peaks (Fig. 3*A*) each giving rise to a charge envelope containing +3, +4, +5, +6, and +7 ions in the mass analyzer (Fig. 3*B*). The ions had an experimental

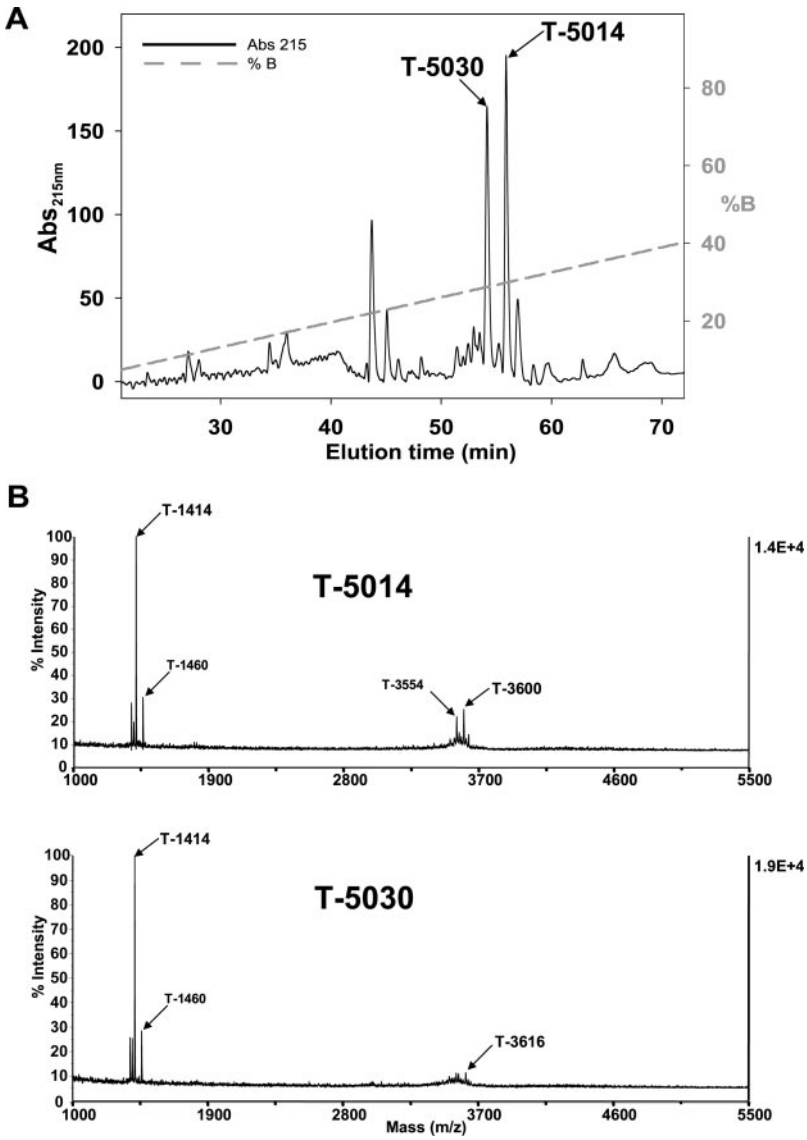

FIG. 4. **The T-5014 complex dissociates into T-1414 peptide and T-3600 peptide under the conditions of MALDI-TOF MS analysis.** *Panel A*, chromatographic analysis of the T_{5K}-complex (Fig. 1) on a Supelcosil C18 reversed-phase HPLC analytical column. *Panel B*, MALDI-TOF spectra obtained from the HPLC-separated T-5030 and T-5014 complexes.

average peptide mass of 5014.0 and 5030.1 and were named T-5014 and T-5030 complexes, respectively. The mass of the T-5014 complex is equal to the sum of the masses of the ions detected by MALDI-TOF MS, T-1414 and T-3600 peptides (Fig. 5). Similarly, the T-5030 complex is equal to the mass of T-1414 and T-3600 peptides plus 16 mass units.

The T-5014 and T-5030 complexes were further characterized by MALDI-TOF MS analyses. The T_{5k}-complex (Fig. 1) was fractionated by reversed-phase HPLC yielding two major components that corresponded to T-5014 and T-5030 complexes (Fig. 4*A*). MALDI-TOF MS analyses (Fig. 4*B*) showed that the T-5014 complex is composed of T-1414 and T-3600 peptides, and that the T-5030 complex is composed of T-1414 and T-3616 peptides. The only difference between T-5014 and T-5030 complexes is the oxidation of methionine in the latter, which may have occurred during chromatography. Thus, the apparent masses of the complexes equal the sum of the masses of the constituent peptides, indicating that interaction of the peptides occurs without a change in mass (non-covalent), or a change in mass (covalent linkage) that is within the experimental error of the measurement for the 5,000 mass range. Moreover, the results establish that the T-5014 and T-5030 complexes dissociate under the conditions of MALDI-TOF MS analyses, a finding that explains why they were not observed in the earlier report (9) that used this method. The presence of fragments T-1460 and T-3554, which are the result of an alternative fragmentation of the tryptic complexes, are indicated in each spectrum (Figs. 1 and 4) and will be addressed below.

The T-5014 complex, isolated by HPLC (Fig. 4*A*), was also characterized by conventional amino acid analysis and Edman degradation. The amino acid composition (Table I) revealed a very close correlation between the experimental and theoretical values based on the sequences of the composite T-1414 and T-3600 peptides, identified by mass spectrometry, including one residue of Hyl in the T-1414 peptide. Amino acid analyses of monomer and dimer subunits also revealed the presence of one Hyl residue in monomer and two residues in the dimer (data not shown). Twenty cycles of Edman degradation revealed the presence of two peptides with sequences of ^{77}NDYSYWLSTPEPMPMSMAPITGENIRPFISR^{107} and ^{204}KPTPSTLK$_{OH}$AGELR^{216}, which correspond to T-3600 and T-1414 peptides (Fig. 5). The latter peptide contained Hyl at the eighth position, corresponding to Hyl^{211}, as suggested by the above mass spectrometry studies. Furthermore, mass spectrometry and Edman degradation analyses indicate that this Hyl is not glycosylated. Moreover, that the T-5014 complex sequenced through 20 cycles without interruption at Met^{93} or Hyl^{211} indicates that the interaction between T-1414 and T-3600 peptides is labile under the conditions of Edman degradation.

Evidence for a Covalent Cross-link in the Tryptic Complex (T-5014)—The chemical nature of the interactions between T-1414 and T-3600 peptides in the T-5014 complex was explored by LC-ESI/MS^3 mass spectrometry, using collision-induced dissociation for fragmentation. The fragmentation generated two modified peptides: T-1460 and T-3554 that differed in mass from the T-1414 and T-3600 peptides observed by MALDI-TOF (see above). Although the modified peptides (T-1460 and T-3554) were much less intense than T-1414 and T-3600 peptides, they were also observed in the MALDI-TOF MS spectra (Figs. 1 and 4). These results suggested that a chemical group of ~46 was transferred from the T-3600 to T-1414 peptide, possibly indicating a covalent cross-link between the two peptides. However, the location of mass changes within the two peptides could not be interpreted with confidence because of the complexity of MS^3 spectra because of the large masses of the peptides. To circumvent this problem, the T_{5k}-complex isolated in Fig. 1 was digested with a second protease to reduce its size for characterization by LC-ESI/MS^3 mass spectrometry.

The large number of prolyl residues in the T_{5K}-complex suggested the use of post-proline endopeptidase for truncation. This enzyme cleaves the peptide bond on the carboxyl side of prolyl residues (13), and it would reduce the T-5014 complex of 44 residues down to 14 residues, as depicted in Fig. 5. The truncated product would be composed of MSMAP (535.2) and $STLK_{OH}AGELR$ (989.6) peptides, which together exist as a complex with a theoretical mass of 1524.8 (designated as the P-1525 complex). The post-proline endopeptidase digestion product was analyzed by LC-ESI/MS^3. Fig. 6*a* (*left panel*) presents a full MS spectrum showing two major ions at *m/z* 509.1 and 762.7 corresponding to the triply and doubly charged forms of the P-1525 complex, respectively. The experimental average mass of the P-1525 complex is 1523.9, approximately 1 mass unit less than the theoretical mass of 1524.8 (Fig. 5), indicating that interaction of the two constituent peptides of the P-1525 complex results in the loss of a single hydrogen.

Further MS/MS analyses of the doubly charged ion of *m/z* 762.6 revealed ions of *m/z* 488.3 and 1036.6 (Fig. 6*a*, *right panel*). These ions differ from the theoretical ones of 536.2 (535.2 + H^+) and 990.6 (989.6 + H^+), calculated for the two constituent peptides of the P-1525 complex (Fig. 5), by values of −48 and +46 mass units, respectively. To obtain structural information about each of these ions and the nature of the 48 and 46 masses, each ion was submitted to a third collision-induced dissociation fragmentation (MS^3) in the instrument. In Fig. 6*b*, the MS^3 spectrum at the *m/z* 488.3 fragment is shown; the fragmentation profile is consistent with the MSMAP peptide sequence, except that the *b* and *y* ion series demonstrate the loss of 48 atomic mass units of Met^{93}. In contrast, in Fig. 6*c*, the fragmentation profile of the ion at *m/z* 1036.6 is consistent with the $SKLK_{OH}AGELR$, but the *y* and *b* ion series demonstrate that Hyl^{211} gained 46 atomic mass units of fragmentation of the P-1525 complex. These fragmentation profiles indicate a loss of a CH_3S- group along with a proton (totaling 48 atomic mass units) from Met^{93} and a gain of a CH_3S- group onto Hyl^{211} and the loss of a proton (totaling 46 atomic mass units).

Overall, the MS results provide compelling evidence that the two peptides of the P-1525 complex are connected by a covalent bond between the side chains of Met^{93} and Hyl^{211}, and that its formation is concomitant with the loss of 1 mass unit. A structure for the cross-link is proposed in Fig. 6*d*, in which the S atom of Met^{93} is covalently linked to the C^{ϵ} atom of Hyl^{211}, forming a sulfonium linkage. Alternatively, the S atom could be attached directly to the N atom on C^{ϵ}, to C^{δ}, or the O atom on C^{δ}, either of which are consistent with the loss of the CH_3S-group from Met^{93} and the gain of this group onto Hyl^{211} upon collision-induced dissociation fragmentation. Based on the x-ray structure of the NC1 hexamer, Than *et al.* (8) proposed a thioether bond that linked S^{δ} of Met^{93} and C^{ϵ} of Lys^{211}, with the concomitant loss of the methyl group of Met^{93} and ϵ-amino group of Lys^{211}; this structure is incompatible with all the mass spectrometry results reported herein, and the presence of Hyl^{211}.

TABLE I
Amino acid composition of T-5014 complex

The T-5014 complex was purified by reversed-phase chromatography as shown in Fig. 4*A*

Amino acid	Number of residues	
	Theoretical	Experimental
Ala	2	2.1
Arg	3	2.9
Asn + Asp	3	3.4
Asn	2	
Asp	1	
Cys	0	0
Gln + Glu	3	3.6
Gln	0	
Glu	3	
Gly	2	3.1
His	0	0
Ile	3	3
Leu	3	3.2
Lys	2	1.1
Hyl	0	0.9
Met	3	2
Phe	1	0.7
Pro	7	7.3
Ser	5	4.7
Thr	4	3.7
Trp	1	
Tyr	2	2
Val	0	0

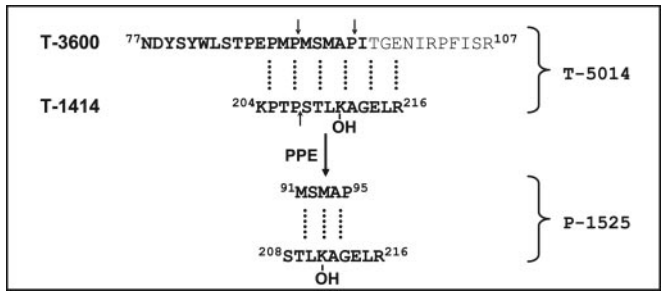

FIG. 5. **Primary structure and location of the T-5014 and P-1525 complexes in the α1 NC1 domain dimer.** The T-3600 peptide corresponds to sequence 77–107 of the α1 NC1 domain, and the T-1414 peptide corresponds to the sequence 204–216 of the α1 NC1 domain with the exception that Lys^{211} is modified to Hyl^{211}. The T-5014 complex, composed of T-3600 and T-1414 peptides, distinguishes dimers from monomers as shown in Fig. 1. The peptide sequences obtained by 20 cycles of automated Edman degradation sequencing of the T-5014 complex are shown in *bold*. Hyl^{211}–Ala^{212} is a potential cleavage site for trypsin; however, in the T-5014 complex, this peptide bond is resistant to cleavage (see below). Digestion of the T-5014 complex with post-proline endopeptidase (*PPE*) generates the P-1525 complex, which is composed of a peptide containing Met^{93}, derived from T-3600 peptide, and a peptide containing Hyl^{211}, derived from the T-1414 peptide. The *arrows* indicate the cleavage sites that generate the P-1525 complex. The mass P-1525 complex is the theoretical value for the two unmodified peptides.

Conditions for Breakage of the Cross-link between Monomers of the NC1 Dimer—The labile nature of the T-5014 complex during the MALDI-TOF MS analyses prompted us to explore the conditions for breaking the cross-link(s) between NC1 monomers of the PBM dimer. In our previous work (9), we

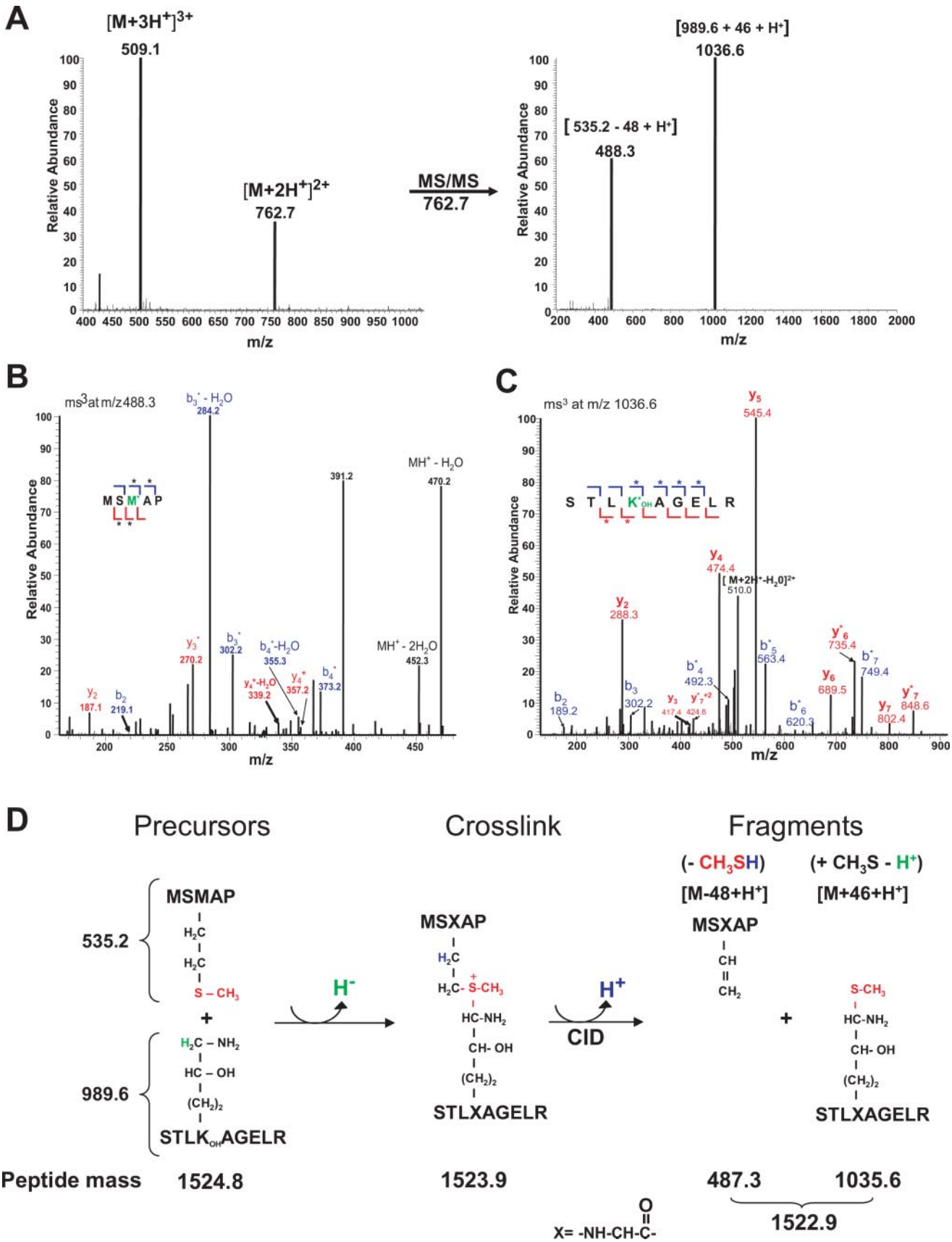

FIG. 6. **LC-ESI/MS^3 analyses of the P-1525 complex demonstrate that Hyl^{211} and Met^{93} are covalently cross-linked.** The T-5014 complex was digested with post-proline endopeptidase (Fig. 5) and analyzed by LC-ESI/MS^3. *A* (*left*) shows the ESI mass spectra of the P-1525 complex constituted by MSMAP and STLK$_{OH}$AGELR peptides. The observed *m/z* values of the doubly $[M + 2H^+]^{2+}$ and triply $[M + 3H^+]^{3+}$ charged ions give an experimental average peptide mass of 1523.9, which is approximately 1 atomic mass unit below the theoretical mass for the P-1525 complex (1524.8). *A* (*right*) shows the MS^2 mass spectra of the doubly charged ion at *m/z* 762.7. The loss of 48 atomic mass units by the MSMAP peptide (*m/z* 536.2) and the concomitant gain of 46 atomic mass units by the STLK$_{OH}$AGELR peptide (*m/z* 990.6) are indicated in the figure between the *squared brackets*. *B* shows that the MS^3 spectrum of the ion at *m/z* 488.3 is consistent with the sequence MSM*AP, where M* indicates the

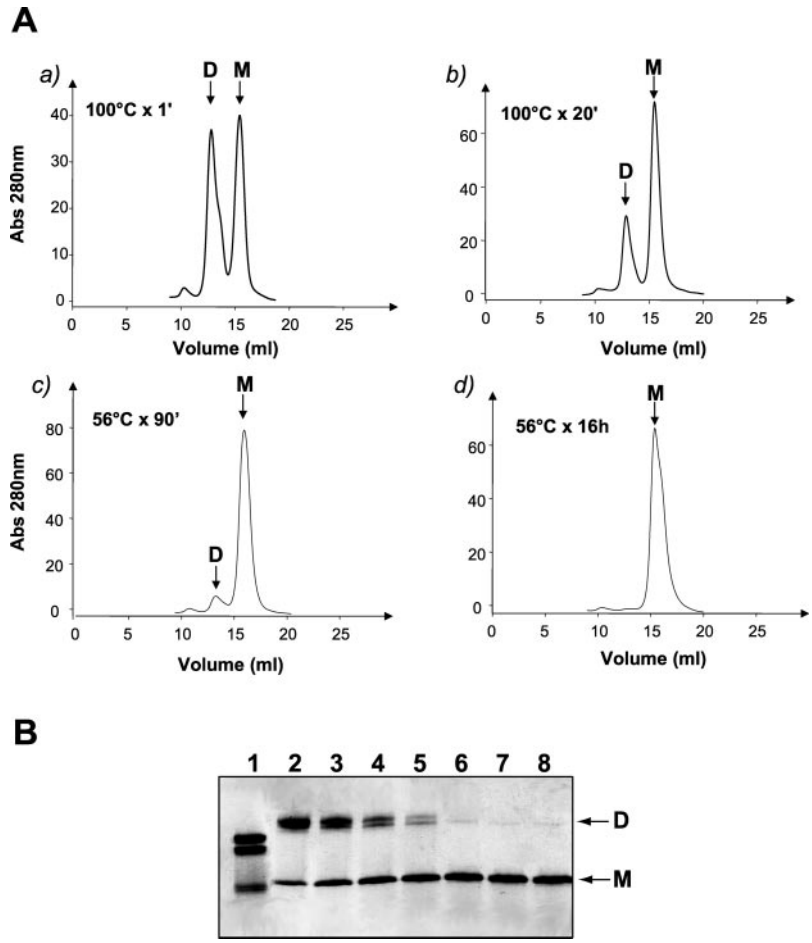

FIG. 7. **The cross-link that connects monomers forming dimer subunits is labile to DTT in the presence of protein denaturants.** *Panel A*, the monomer and dimer ratio was determined by size-exclusion HPLC on a TSK SW_{xl}3000 HPLC column, equilibrated with 4 M GdnHCl. PBM hexamers were reduced in 0.2 M Tris-HCl, pH 8.5, containing 4 M GdnHCl and 25 mM DTT by the indicated time and temperature. The samples were alkylated with 50 mM iodoacetamide and incubated in the dark for 30 min before injection. *Panel B*, SDS-PAGE analyses: time course experiment showing the monomer (M) to dimer (D) ratio after incubating PBM hexamers at 80 °C for 0, 15, 30, 45, 60, 90, and 120 min (*lanes 2–8*, respectively) in Laemmli buffer (2% SDS), which included 100 mM DTT. A control sample (*lane 1*) where the hexamer was heated for 2 h at 80 °C without DTT did not significantly alter the dimer:monomer ratio, showing that the conversion of dimers into monomers occurs only in the presence of the reducing agent.

established that PBM NC1 hexamers dissociate into dimer and monomer subunits (80/20 ratio) under denaturing conditions by gel-filtration chromatography (4 M GdnHCl and pH 3.0) and SDS-PAGE. In the present study, we extended those studies to investigate the effect of DTT and denaturant as a function of temperature and incubation time on hexamer dissociation. Fig. 7*A* shows the gel-filtration dissociation profiles of hexamer treated with DTT in GdnHCl under different incubation time and temperature conditions. The dimer:monomer ratio decreases to 50:50 after incubation of hexamers at 100 °C for 1 min in the presence of DTT and GdnHCl (*panel a*). The amount of dimer decreases even more, a change of ratio from 80:20 to 30:70 when the incubation at 100 °C is extended to 20 min (*panel b*). Similarly, incubation of hexamers for 90 min at 56 °C changes the dimer:monomer ratio to 10:90 (*panel c*), while extending the incubation time to 16 h, in the same conditions, achieves complete conversion of dimers into monomers (*panel d*). These experiments demonstrate that DTT in the presence of denaturant converts dimers into monomers, breaking the cross-link that hold dimers together.

The dissociation of PBM hexamer was also studied by SDS-PAGE with the samples treated with DTT and SDS. Fig. 7*B* shows a decrease in mobility of the monomer and dimer bands as expected by the reduction of intramolecular disulfide bonds (*lanes 1* and *2*). In addition, a gradual conversion of dimers into monomers (*lanes 2–8*) as a function of incubation time is clearly demonstrated. Importantly, incubation of hexamers for 2 h at 80 °C in the absence of DTT (*lane 1*) did not change the 80:20 dimer:monomer ratio. Thus, DTT appears to exert two effects: the reduction of disulfide bonds and the breakage of the Met-Hyl cross-link.

Mass spectrometry analysis of the breakage of dimers into monomers can also provide information about the location and nature of the cross-link. In the dimer, the Hyl^{211}–Ala^{212} bond is resistant to trypsin cleavage, resulting in the excision of the T-1414 peptide with an intact Hyl^{211}–Ala^{212} bond (Fig. 5); such bonds are known to be susceptible to trypsin cleavage except when Hyl is modified by a carbohydrate unit (14). This poses the questions of whether the Hyl^{211}–Ala^{212} bond is resistant or susceptible to cleavage when present in the monomer, and

loss of 48 atomic mass units from Met^{93}. The *asterisk* (*) above the *b* (⌉) and *y* (⌊) ions indicates the loss of 48 atomic mass units from the expected mass value of the unmodified fragments. *C* shows that the MS^3 spectrum of the ion at *m/z* 1036.6 is consistent with the sequence $STLK^*_{OH}AGELR$, where K^*_{OH} represents the gain of 46 atomic mass units by the Hyl^{211} residue. The *asterisk* (*) above *b* (⌉) and *y* (⌊) ions indicates the gain of 46 atomic mass units the predicted mass value of the unmodified fragments containing the Hyl^{211} residue. *D* presents a proposed structure for the covalent linkage between the peptides containing Hyl^{211} and Met^{93} based on the mass spectrometry results. The S atom of Met^{93} is covalently linked to the C^{ϵ} atom of Hyl^{211} forming a sulfonium ion. Alternatively, the S atom could be attached directly to the N atom on C^{ϵ}, to C^{δ}, or the O atom on C^{δ} of Hyl, either of which are consistent with all the LC-ESI/MS^3 results. Note that the difference in mass between the precursors (1525) and the cross-linked peptides (1524) is 1 mass unit, represented by the loss of H^- in each alternative. The novel cross-link is termed *S*-hydroxylysyl-methionine to denote the linkage via the sulfur atom. The structures for the fragments generated by collision-induced dissociation of the P-1525 complex are shown at the *right* of the cross-link. A thioether bond was previously proposed by Than *et al.* (8) that linked S^{δ} of Met^{93} and C^{ϵ} of Lys^{211}, but with the concomitant loss of methyl group of Met^{93} and ϵ-amino group of Lys^{211}, a structure that is incompatible with all the mass spectrometry results reported herein.

whether it becomes susceptible after breakage of dimer into monomer by treatment with DTT. Thus, the status of the Hyl^{211}–Ala^{212} bond was explored in two kinds of monomers: monomers that were derived from hexamers and fractionated by gel-filtration chromatography (Fig. 7*A*, *panel b*), and monomers that were derived from the dimers (Fig. 7*A*, *panel b*) followed by treatment with 2% SDS plus 100 mM DTT and incubated for 2 h at 80 °C.

This objective was accomplished using SDS-PAGE to separate dimers and monomers, in-gel digestion of components with trypsin to release peptides, and LC-ESI/MS/MS analyses of tryptic peptides. The data sets were interrogated for the presence of the T-1414 peptide, which contain the intact Hyl^{211}–Ala^{212} bond, and T-887 peptide, which corresponds in mass to a peptide with the sequence of $KPTPSTLK_{OH}{}^{211}$ and that indicates cleavage of the Hyl^{211}–Ala^{212} bond (Fig. 8). The T-1414 peptide was present in the dimer (*lane 1*), consistent with previous MS analyses, but not in the monomer (*lane 2*). Conversely, the T-887 peptide was present in the monomer but not in the dimer. The T-887 peptide was also present in monomer (*lane 3*) that had been treated at elevated temperatures, but with DTT. Moreover, the monomer that was derived by DTT treatment of dimer (*lane 4*) also revealed the presence of the T-887 peptide, but the complete absence of the T-1414 peptide. These results indicate that the Hyl^{211}–Ala^{212} bond is resistant to trypsin cleavage in the dimer subunit, but susceptible to cleavage in the monomer subunit, and that the resistance is broken by treatment with DTT. The findings are consistent with a covalent cross-link involving Hyl^{211} rendering the Hyl^{211}–Ala^{212} bond resistant to trypsin cleavage. Moreover, the presence of Hyl^{211} in both M- and D-hexamers suggests that the post-translational modification of Lys^{211} in monomers is necessary but not sufficient for cross-linking of monomers to form dimers.

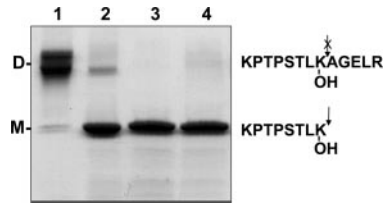

FIG. 8. **Disruption of the reinforced interactions/cross-link of dimer subunits renders the Hyl^{211}--Ala^{212} peptide bond susceptible to trypsin cleavage.** For comparison, the dimer and monomer subunits, as shown in Fig. 7*A* (*panel b*), were subjected to SDS-PAGE and in-gel digestion by trypsin, and the tryptic products analyzed by LC-ESI/MS/MS. As anticipated, the dimer (*lane 1*) yielded T-1414 peptide containing Hyl^{211} as described in the legend to Fig. 5. In contrast, the monomers (*lane 2*) yielded T-887 peptide, which correspond to the sequence $KPTPSTLK_{OH}{}^{211}$, indicating the presence of Hyl^{211} as well as the cleavage of the Hyl^{211}-Ala^{212} peptide bond in monomers. In *lane 3*, the monomer was incubated at 80 °C for 2 h in Laemmli buffer (2% SDS) containing 100 mM DTT, followed by electrophoresis and in-gel digestion with trypsin, and in *lane 4*, the dimer (same as *lane 1*) was treated in like manner. LC-ESI/MS/MS analysis showed that DTT treatment of monomer yielded the same T-887 peptide as the untreated monomer (*lane 2*); however, DTT treatment of dimer not only converted it to monomer but also altered the tryptic product from the T-1414 peptide to the T-887 peptide, indicating that conversion of dimers to monomers renders the Hyl^{211}–Ala^{212} peptide bond susceptible to trypsin cleavage.

DISCUSSION

In a recent study (9), we presented evidence for the existence of two distinct kinds of hexamers, $M^{\alpha1\alpha2}$-hexamers composed exclusively of monomers, and $D^{\alpha1\alpha2}$-hexamers composed exclusively of dimers. The two extremes reflect a process that reinforces/cross-links the interaction of monomer subunits forming D-hexamers. The proportion of M- and reinforced D-hexamers indicate that the collagen IV network of PBM is a more stable structure than that of the lens capsule basement membrane, a feature that may be an important determinant of biological function. The reinforcement is not unique to the $\alpha1\alpha1\alpha2$(IV) network, but also occurs in the $\alpha3\alpha4\alpha5$(IV)- and $\alpha1\alpha1\alpha2$-$\alpha5\alpha5\alpha6$(IV) networks (1, 3, 4).

In the present study, the location and nature of the reinforcement/cross-link site of dimers of $D^{\alpha1\alpha2}$-hexamers were investigated using trypsin digestion as a strategy to excise the site and using mass spectrometry for characterization. Fortuitously, a structural feature distinguishing dimers from monomer subunits could be excised as a low molecular weight complex that was easily purified for chemical and physical characterization. The tryptic complex is composed of two short peptides, comprising residues 77–107 and 204–216 of an α1-NC1 monomer. The two peptides, respectively, contain Met^{93} and Lys^{211} post-translationally modified to Hyl^{211}, and they correspond to one of the regions that are in close proximity at the trimer-trimer interface of the NC1 hexamer, as defined previously from the crystal structure (7–9). That the complex is derived exclusively from dimer subunits of $D^{\alpha1\alpha2}$-hexamers, and its primary structure corresponds to a region that connects the trimer-trimer interface, provides the first chemical evidence for the location of the reinforcement/cross-link site (Fig. 9). Its location is in agreement with that proposed by Than *et al.* (8) based on connectivity observed by x-ray crystallography, but differs with respect to the presence of Hyl^{211} instead of Lys^{211}.

The chemical nature of the reinforcement/cross-link was elucidated by mass spectrometry of a truncated form of the tryptic complex. A second digestion with post-proline endopeptidase truncated the 44 amino acid residues down to 14, rendering the complex amenable to LC-ESI/MS^3 mass spectrometry. The fragmentation patterns, the loss the CH_3S- group from Met^{93} and its transfer to Hyl^{211}, provided compelling evidence that the two peptides of the smaller complex are connected by a covalent bond between the side chains of Met^{93} and Hyl^{211}, and that its formation is concomitant with the loss of 1 mass unit, relative to the masses of unmodified Met^{93} and Hyl^{211}. The proposed structure, shown in Figs. 6*d* and 9, is a sulfonium ion in which the S atom of Met^{93} is covalently linked to the C^{ϵ} atom of Hyl^{211}. Alternatively, the S atom could be attached directly to the N atom on C^{ϵ}, to C^{δ}, or the O atom on C^{δ} of Hyl, either of which are consistent with all the LC-ESI/MS^3 results. The sulfonium ion linkage is consistent with the susceptibility of the cross-link to cleavage by DTT (Figs. 7–9), because methionine sulfonium ions undergo cleavage by sulfhydryl agents (15). Of particular note, the linkage is also susceptible to cleavage by the conditions of Edman degradation and MALDI-TOF analysis; the latter explains why the cross-link was not observed by MALDI-TOF analysis of dimer subunits in our previous study. Several amino acid residues, including methionine, are susceptible to radiation damage by synchrotron x-rays (16). The breakage in methionine is known to occur at the CH_3S- group. This may be the reason why we did not detect the cross-link involving the Met^{93} residue in the crystal structure (9) and why it is difficult to model accurately even at 1.5-Å resolution.

The novel cross-link is termed *S*-hydroxylysyl-methionine to denote a sulfur atom connection between Hyl and Met. At least two events occur in the formation of the cross-link: the post-translational hydroxylation of Lys^{211} to Hyl^{211} within the NC1 domain during the biosynthesis of α-chains; and the connection of Hyl^{211} to Met^{93} between the trimeric NC1 domains. The cross-link connects two adjoining triple-helical protomers, reinforcing the stability of collagen IV networks.

The presence of hydroxylysine 211 in the NC1 domain is a

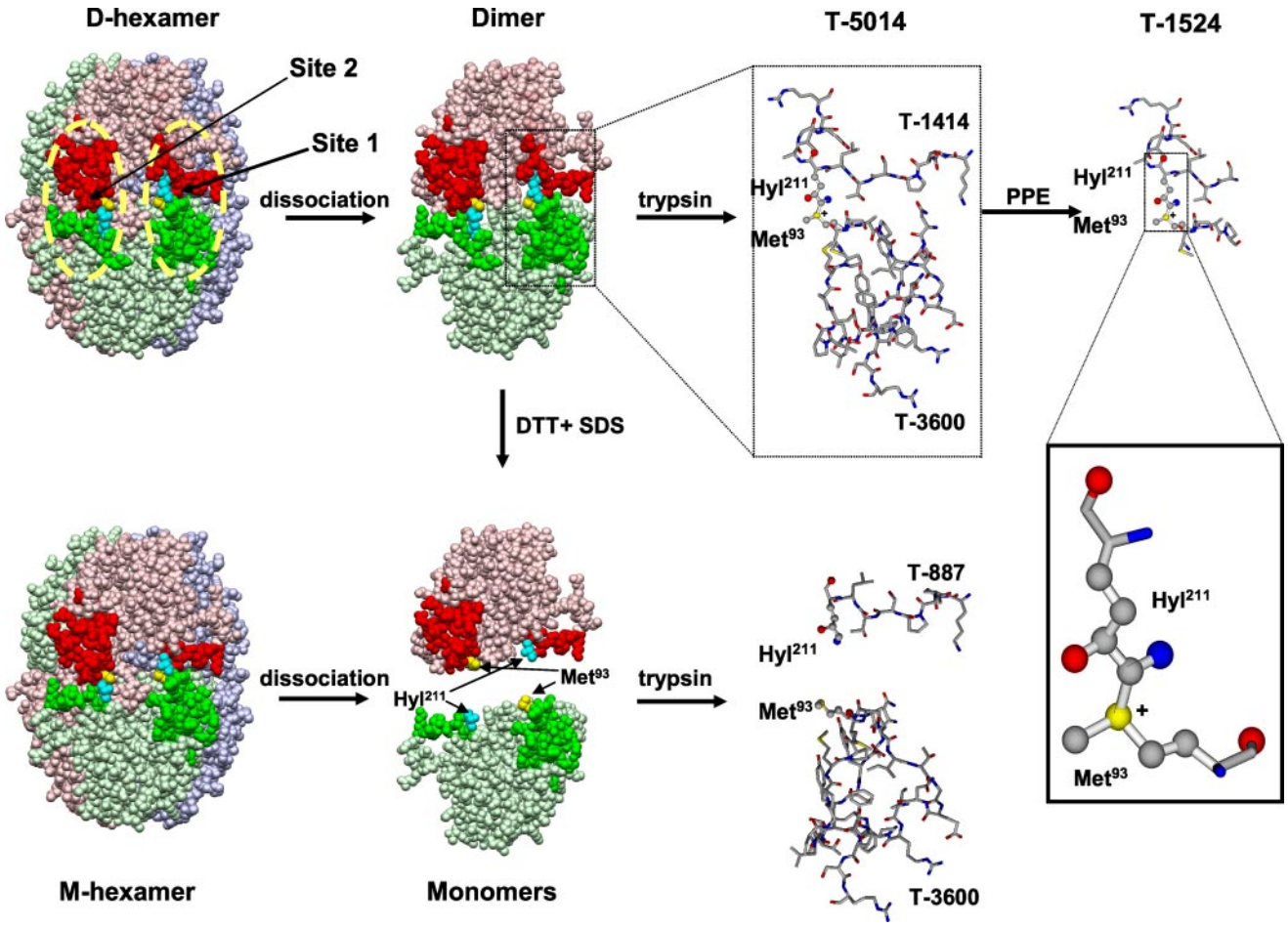

FIG. 9. **Summary of findings regarding the location and nature of the reinforcement/cross-link site.** The *top panel* shows the space-filling model for the reinforced/cross-linked D-hexamer (*left*) comprised of two trimeric caps, each composed of two α1 monomers and one α2 monomer. The juxtaposition of two opposing α1 NC1 monomers at the hexamer interface generates two sites of reinforcement: site 1 and site 2, which are defined by the tryptic 5014 complex composed of T-3600 (*red*) and T-1414 (*green*) peptides. The side chain of Met^{93} is colored in *gold*, and the side chain of Hyl^{211} is colored in *cyan*. In site 2, the peptide (not the side chains) colors are reversed. Under denaturing conditions, the D-hexamer dissociates into dimer subunits (*middle*). The dimer upon trypsin digestion releases T-5014 complex (*right*), composed of the T-3600 peptide (31 residues, *red*) and T-1414 peptide (13 residues, *green*), which constitutes the reinforcement site. The T-1414 peptide contains a post-translational modification in which Lys^{211} is converted to Hyl^{211}. For mass spectrometry analyses to determine the chemical nature of the cross-link, the T-5014 complex was truncated by digestion with post-proline endopeptidase (*PPE*), which generated the P-1525 complex composed of the MSMAP peptide covalently cross-linked to the $STLK_{OH}AGELR$ peptide. The side chains of Met^{93} and Hyl^{211} are covalently connected by a sulfonium ion linkage between S^{δ} of Met^{93} and C^{ϵ} of Hyl^{211}, as presented in Fig. 6*d*. A magnification of the proposed *S*-hydroxylysyl-methionine cross-link is shown. The *bottom panel* illustrates the non-reinforced/non-cross-linked M-hexamers (*left*), which dissociates into NC1 monomers (*middle*) upon denaturation. In this case trypsin digestion generates T-3600 and T-887 peptides that are not cross-linked in the hexamer. T-887 peptide, derived from the monomers, is a truncated version of T-1414 peptide derived from dimers. The structural relationship of these peptides indicates that in the dimer the Hyl^{211}–Ala^{212} bond is blocked to trypsin cleavage, but in the monomer this bond is susceptible to cleavage. This difference in susceptibility to trypsin cleavage provides independent support for the location of a cross-link at Hyl^{211}, and the difference is consistent with a covalent linkage between Met^{93} and Hyl^{211}. The presence of Hyl^{211} in both M- and D-hexamers suggests that the post-translational modification of Lyl^{211} in monomers is necessary but not sufficient for cross-linking of monomers to form dimers.

novel feature. Hyl is a post-translational modification of lysine residues that typically occurs in *X*-Lys-Gly triplets in the collagenous domain of various types of collagens and collagen-like proteins (12). Hyl was previously noted in the preparation of NC1 domains (17), but it was thought to be a contaminant from the collagenase digestion of the collagenous domain or a residue in the two Gly-X-Y triplets at the NH_2 terminus of the isolated NC1 domains (Fig. 5, Ref. 18). In certain cases, hydroxylation occurs in nonhelical peptides with sequences of *X*-Lys-Ala(Ser) at the end of collagen I chains that are involved in aldehyde-derived cross-links (19). This consensus sequence is identical to the X-Lys^{211}-Ala sequence in the T-1414 peptide.

Hyl residues have two important functions. They are essential for the stability of the intermolecular collagen cross-links, and their hydroxyl groups serve as attachment sites for the monosaccharide galactose or the disaccharide glucosyl-galactose (20–22). The carbohydrate units influence the lateral packing of fibril-forming collagen molecules into fibrils and may facilitate the assembly of the 7 S domain of collagen IV (23). In a recent study, lysyl hydrolysase-3 was shown to be essential for the assembly of collagen IV networks, presumably because of the absence of the hydroxylysine-linked carbohydrates (24). Conceivably, such carbohydrate units attached to Hyl^{211} might prevent the self-assembly of collagen IV protomers into networks within the intracellular environment, but upon deglycosylation in the extracellular space, network assembly could take place. In a related example, the NH_2- and COOH-terminal propeptides of fibril-forming collagens prevent assembly of fibrils until removed by proteases in the extracellular environment.

Finally, the *S*-hydroxylysyl-methionine cross-link, likely occurs in all three collagen IV networks ($\alpha1\alpha1\alpha2$, $\alpha3\alpha4\alpha5$, and $\alpha1\alpha1\alpha2$-$\alpha5\alpha5\alpha6$ networks). Each NC1 domain of the six human α-chains exist as both monomers and dimers: the α1-like mono-

mers form α1-α1, α1-α5, and α3-α5 dimers, and the α2-like monomers form α2-α2, α2-α6, and α4-α4 dimers (3, 4), indicating the presence of a cross-link. As noted previously by Than *et al.* (8), the sequences encompassing Met^{93} and Lys^{211} are invariant among all six human α-chains, suggesting that the same post-translational modifications could occur in each chain. Of particular importance, the reinforcement/cross-link of the α3α4α5 network was recently found to sequester B-cell epitopes within the NC1 hexamer, rendering them inaccessible to pathogenic autoantibodies in patients with Goodpasture syndrome. Thus, the cross-link represents a novel molecular mechanism for establishing immune privilege (25).

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